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13. ABSTRACT (Maximum 200 words) When deprived of adhesion to a solid substrate, fibroblasts are unable to proliferate and arrest in the G1 phase of the cell cycle. We have shown that there is a similar adhesion -responsive G1 restriction point in the immortalized human mammary epithelial cells line, 184A1N4. Unlike normal cells, transformed cells gain the ability to bypass this integrin-mediated control of proliferation and to grow in an anchorage-independent manner. We are examining the effect of c-Myc overexpression, which frequently occurs in breast cancer, in the adhesion-dependent regulation of the G1-S phase transition. A1N4 cells overexpressing c-Myc (A1N4-Myc) synchronized in G0/G1 phase by EGF withdrawal are able in suspension culture to progress through G1 phase and enter S phase upon EGF stimulation. Comparison at the molecular level of A1N4 and A1N4-Myc cells indicates that whereas in the non-adherent A1N4 cells the cyclin E-CDK2 complex is only present in its inactive form, associated with the p27 inhibitor, overexpression of c-Myc in these cells is sufficient to induce cyclin E-CDK2 activation and pRb phosphorylation. The upregulation of CDK2 activity is the result of a specific downregulation of total cellular levels of the cyclin dependent kinase inhibitor p27, and thus a decrease association of p27 with CDK2. We further show that cells overexpressing c-Myc have a higher turnover rate of p27 degradation through the ubiquitin-proteasome pathway pointing to a new mechanism by which c-Myc induces cyclin E-CDK2 activation and progression into S phase in cells deprived of adhesion.				
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FOREWORD

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INTRODUCTION

To proliferate, normal cells require two types of extracellular signals: hormonal/growth factor stimuli and adhesion to the extracellular matrix (ECM). When deprived of attachment to a solid substrate, even in the presence of growth factors, normal cells are unable to replicate their DNA, and they arrest in the G1 phase of the cell cycle (1,2). A hallmark of transformed cells, which correlates with tumorigenicity, is the loss of anchorage dependent growth. As cells become malignant, they gain the ability to bypass the extracellular matrix control of cell proliferation.

Recent studies have linked cell adhesion to key regulatory molecules of cell cycle progression: the cyclins and their associated cyclin-dependent kinases (cdk). In mouse and human fibroblasts, loss of adhesion results in a down regulation of expression of cyclin D1 (1,3) and cyclin A (4), an inhibition of cyclin E-cdk2 activity through its association with the cdk inhibitors p21 and p27, and a hypophosphorylation of the tumor suppressor protein Rb-1 (3,5).

A potential and still controversial link between integrin-mediated cell adhesion and upregulation of cyclin expression and cdk activity is the c-Myc proto-oncogene, which is frequently overexpressed in breast cancer. Reattachment of suspension-arrested BALA/3T3 fibroblasts is sufficient to induce expression of c-Myc mRNA, independently of growth factors (2). Conversely, a three-fold decrease in Myc mRNA is observed in the presence of growth factors, when actively growing Rat1a fibroblasts are deprived adhesion, compared to cells allowed to attach to substrate (6). However, Böhmer reported no difference in Myc mRNA levels when human fibroblasts synchronized in G0 by serum starvation were submitted to mitogen stimulation either as a monolayer or in suspension (1).

Myc is a transcription factor containing a helix-loop helix leucine zipper domain that enables dimerization with Max, and specific binding of the heterodimer to the E box DNA motif (7). Myc activates the transcription of a number of genes involved in cellular proliferation. Its precise target genes still need to be defined. Experiments altering Myc expression indicates that Myc is required for progression through the G1 phase of the cell cycle. Activation of a Myc-inducible expression construct in quiescent fibroblasts is sufficient to drive the cell through the entire cell cycle (8). Similarly, overexpression of Myc prevents growth arrest normally induced by growth factor withdrawal (9). Conversely, inhibition of Myc expression with antisense oligonucleotides prevents cell proliferation and arrests the cells in late G1 (10), and diminution of Myc levels by disruption of one of the myc alleles lengthens the G0 to S transition (11).

The mechanism by which Myc mediates progression through the cell cycle is the subject of current investigation and needs to be further elucidated. Myc has been shown to induce cyclin E and cyclin A expression (12), as well as the activity of the cyclin E-cdk2 complex, presumably by triggering the release of the kinase inhibitors p27 and p21 (13,14), an effect strongly reminiscent of the

one caused by cell adhesion. Cdk2 is further subject to both positive and negative regulation by direct phosphorylation. Thus, in addition to the displacement of the cdk inhibitors, cdk2 activation requires the removal of inhibitory phosphate groups by cdc25 phosphatase (15). Myc might also be implicated in this activation step since it induces the expression of cdc25 (16).

In addition to the activation of the cyclin E-cdk2 complex, cellular adhesion also results in the activation of cyclin D-cdk4, another complex essential for progression through the G1 restriction point. Since cyclin D1 cooperates with Myc in transforming B cells (17), and cyclin D1 and Myc complement each other in rescuing CSF receptor-deficiency in fibroblasts (18), Myc most likely does not directly induce cyclin D1, and several different pathways are probably implicated in transducing the ECM regulatory signals to the G1 cyclin-CDK complexes. Whereas cyclin D1 might first be induced in adherent cells by the ras-MAPK pathway, independently of Myc, the src pathway might result in an activation of Myc, which specifically regulates the activity of cdk2 in late G1.

To date, most of the studies on anchorage dependent growth regulation have been performed only on fibroblasts. Unlike fibroblasts, which are embedded in a three dimensional extracellular matrix rich in collagen I and fibronectin, proliferating mammary alveolar epithelial cells are resting upon a basement membrane composed predominantly of laminin and type IV collagen, which separates them from the stroma. Primary mammary epithelial cells have been shown to receive specific survival signals from basement membrane matrix which are not provided by attachment to plastic or type I collagen (19). Hence, epithelial cells may differ from fibroblasts in their interaction with the extracellular matrix, and the control of cell growth by the ECM may also vary. We have proposed to study whether in human mammary epithelial cells, Myc is a link between the ECM adhesion-dependent growth and cell cycle regulatory machinery.

BODY

To approach the question of whether cell adhesion to the ECM is required for c-Myc induction (Aim 1 of the proposal) we first wanted to determine c-Myc protein levels by western analysis. We initially had some difficulties to detect c-Myc due to a lack of an antibody working in our conditions. At that time we had available in the laboratory all the reagents required to perform the experiments proposed in Aim 2; we therefore decided to first address Aim 2 of the original proposal that was initially scheduled for month 26-38. We now have available a satisfactory antibody and the experiments proposed in Aim 1 will be performed this coming year.

Aim 2: To characterize the role of Myc in mediating anchorage-dependent regulation of cyclin-dependent kinase activity.

- a. Investigate whether cell anchorage activates two parallel pathways, one Myc-independent leading to the expression of cyclin D1, the second involving Myc activation of cyclin E-cdk2 activity.*
- b. Define how Myc induces cdk2 activation, focusing on cyclin E expression, p21 and p27 expression, inhibitor association with the cyclin-cdk2 complex, and regulation of cdc25 phosphatase.*

Since we have not yet shown that c-Myc expression is modulated by cell adhesion, we shifted our hypothesis to the following: Deregulated expression of c-Myc enables mammary epithelial cells to progress through the anchorage-dependent G1 check point. This Myc induced G1-S transition is achieved through the activation of G1 cyclin-CDK complex due to the down regulation of CDK inhibitors.

Our preliminary results using flow cytometry have shown that the immortalized mammary epithelial cell line 184A1N4 (20), can be synchronized and growth arrested in an G0/G1 stage of the cell cycle by EGF withdrawal. Upon readdition of EGF, cells in monolayer progress into S and G2 phase of the cell cycle. However, cells stimulated with EGF in suspension culture using agarose coated dishes were unable to enter S phase. In the contrary, A1N4 cells stably transfected c-myc under a constitutive promoter (A1N4-Myc cells) (21) are able to replicate their DNA in suspension culture.

Those results are further supported at the molecular level by western analysis of the phosphorylation state of the retinoblastoma protein, Rb (Figure 1). Whereas Rb could only be detected in its hypophosphorylated form in non-adherent A1N4 cells, hyperphosphorylated forms of Rb are present in the Myc transfectants. The differential phosphorylation of pRB in the two cell lines confirms that unlike the A1N4 which are blocked in G1, A1N4 cells overexpressing Myc cells progress through the G1-S transition.

We therefore asked whether Myc is able to induce the expression and activity of the various G1 cyclin-cdk complexes in an adhesion independent manner. In all the experiments described below, A1N4 and A1N4-Myc cell lines were compared both in suspension and in monolayer. Both cell lines were treated in parallel. Cells were synchronized in G0/G1 by EGF starvation and then restimulated with EGF as monolayer or suspension culture. Total cell lysates or total RNA were prepared from quiescent (control), adherent and suspended cells at various times following EGF stimulation.

We first examined the cyclin D-CDK4 complex. As expected, CDK4 protein levels are constant in both A1N4 and A1N4-Myc cells whether they are quiescent, stimulated by EGF in monolayer or suspension. Cyclin D transcription has been shown to be down regulated in cells deprived of adhesion, leading to a decrease in cyclin D-CDK4 kinase activity. Northern blot analysis indicates that, although in suspension culture a lag period is required for the EGF-induced cyclin D transcriptional upregulation to occur, by 12 hours equivalent levels of transcripts can be detected in adherent and non-adherent A1N4 cells. Furthermore, a similar pattern of cyclin D mRNA expression is observed in cells overexpressing Myc. In addition, *in vitro* kinase assay where the cyclin D-CDK4 complex was immunoprecipitated with a CDK4 antibody and incubated with [γ^{32} ATP] and recombinant pRB as a substrate, showed similar levels of cyclin D-CDK4 kinase activity in non-adherent A1N4 and A1N4-Myc cells. We therefore concluded that cyclin D-CDK4 activity is still present in non-adherent A1N4 cells and that overexpression of c-Myc does not upregulate CDK4 activity.

We thus examined the cyclin E-CDK2 complex (Figure 2). No kinase activity using histone H1 as a substrate was detectable in *in vitro* kinase assays performed for the cyclin E-CDK2 complex immunoprecipitated from A1N4 cells in suspension cultures (Figure 2C). In contrast, non-adherent A1N4 Myc cells showed the same level of CDK2 kinase activity as adherent cells. To further characterize the molecular mechanism inducing the adhesion-independent activation of cyclin E-CDK2 complex in Myc overexpressing cells we analyzed by western blot the cellular levels of cyclin E, CDK2 and the CDK inhibitors p21 and p27 (Figure 2A). There is a down regulation of cyclin E protein in A1N4 suspension culture which is not observed in A1N4 -Myc cells that maintain in suspension cyclin E levels similar to adherent cells. Total CDK2 levels were not modulated but the phosphorylation of the kinase changed drastically. The threonine 160 phosphorylated form which correspond to the catalytically active CDK2 kinase can be visualized on SDS-PAGE as a faster migrating form. In suspended A1N4 cells CDK2 is present exclusively in its hypophosphorylated form. However phosphorylated CDK2 is detected in non-adherent A1N4-Myc cells. Finally, p27 is strongly upregulated in non-adherent A1N4 cells, but is only detectable at very low level in the total cell lysate from suspension culture of A1N4 overexpressing c-Myc. No variation in expression of the other potential CDK2 inhibitor, p21, was detected. As expected, co-immunoprecipitation of p27 with

a CDK2 antibody showed that whereas p27 is associated with the CDK2 complexes in non-adherent A1N4 cells, the CDK2 complexes in non-adherent A1N4-Myc cells are mostly free of p27 (Figure 2B). Together with the upregulation of cyclin E expression and CDK2 phosphorylation, decreased association of the CDK inhibitor p27 with the CDK2 complex explains the increased CDK2 activity in non-adherent A1N4 cells overexpressing c-Myc.

In our system, the CDK2 kinase activity observed in Myc-overexpressing cells is correlated with a decrease in p27 steady state level. The literature indicates that p27 can be regulated both at the transcriptional and post-transcriptional level. Under certain conditions p27 is rapidly degraded by the proteasome-ubiquitin pathway. We did not detect any significant variation in total p27 mRNA by Northern blots (Figure 3A). To determine whether p27 levels were regulated by a post-transcriptional mechanism, p27 protein stability was analyzed by pulse chase experiments (Figure 3B). A1N4 and A1N4-Myc cells in suspension were labeled for one hour with [³⁵S]methionine and then chased in non-radioactive medium for up to 120 min. Our results shows that the half life of p27 in suspended A1N4 cells is about 2 hours. In contrast, the half life of p27 in cells overexpressing Myc is decreased to less than 40 min. To examine whether the increased turnover rate observed in A1N4-Myc cells is caused by p27 degradation through the proteasome-ubiquitin pathway we treated A1N4 cells in suspension culture with the proteasome inhibitor ALLN (Calbiochem) (Figure 3C). Since ALLN peptide aldehyde also inhibits calpains and cathepsin we also treated the cells with a control peptide, ALLM (Calbiochem), which has an equivalent inhibitory affinity for calpains and cathepsin, but has no effect on the proteasome. Incubation with proteasome inhibitor ALLN specifically lead to a three fold increase in p27 protein, indicating that proteasome dependent degradation contribute to the downregulation of p27 in non-adherent A1N4 cells overexpressing c-Myc.

All our present results have been obtained using cells stably transfected with c-Myc. We therefore cannot exclude at the present the possibility that our findings are due to selection. In order to implicate c-Myc more directly in the ability of A1N4-Myc cells to ignore the anchorage-dependent G1-S check point, we are currently using c-Myc anti-sense. Another alternative approach considered is to confirm the results described above using c-Myc transient transfections.

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APPENDICES

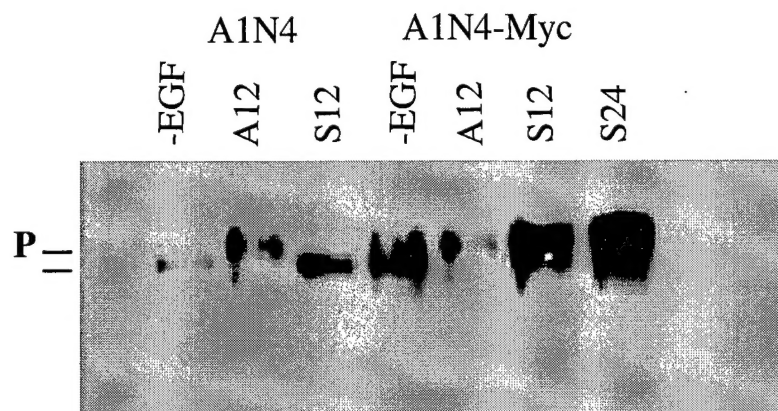


Figure 1: pRb phosphorylation in adherent and non-adherent A1N4 and A1N4-Myc cells. Cells were synchronized by EGF withdrawal (-EGF) for 48 hours and then restimulated with EGF for 12 hours as monolayers (A12) or suspension culture (S12).

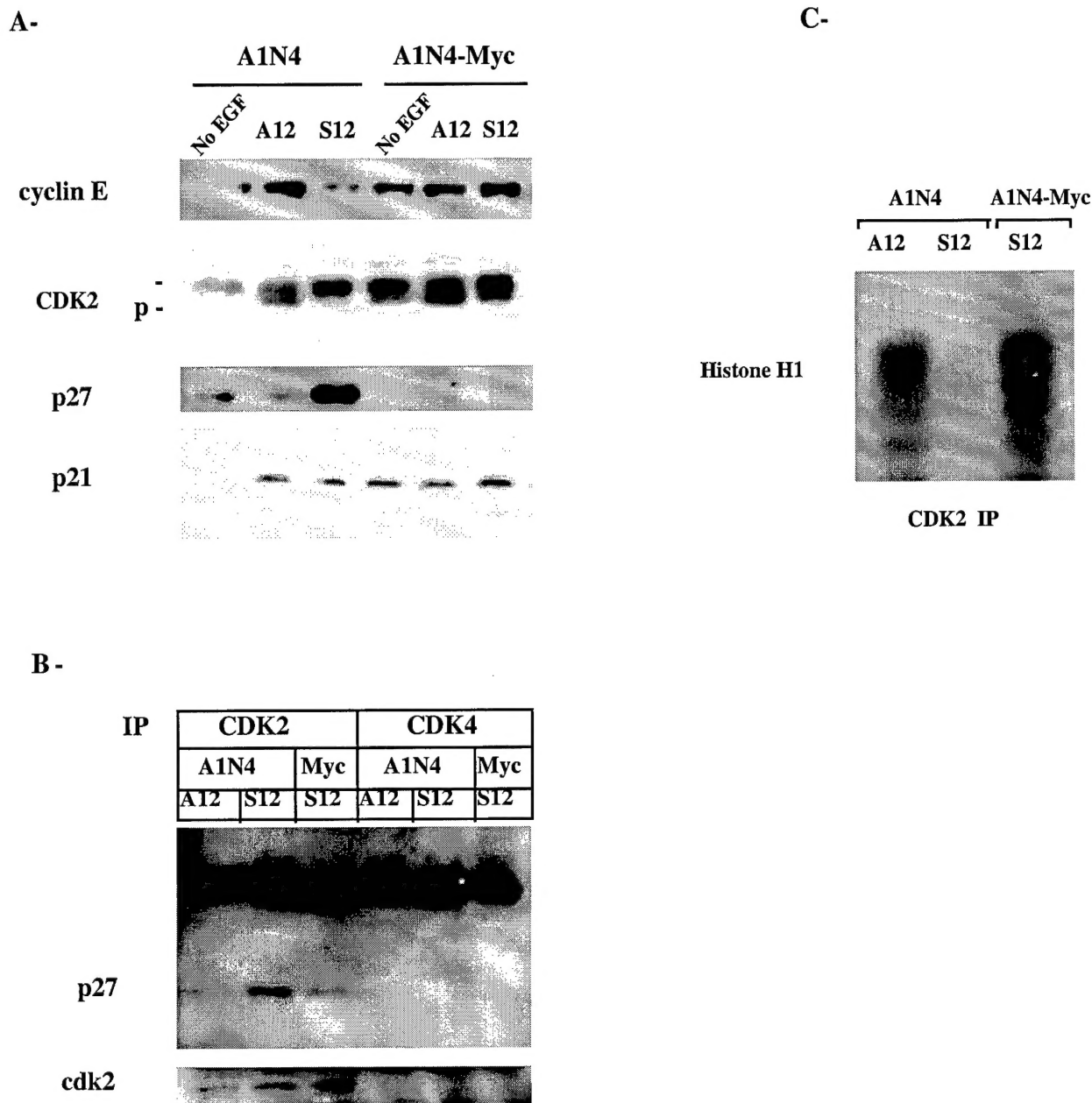
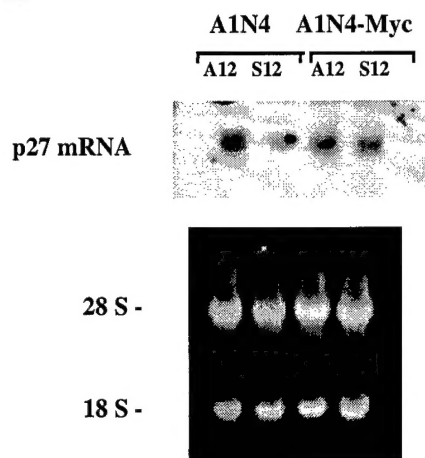
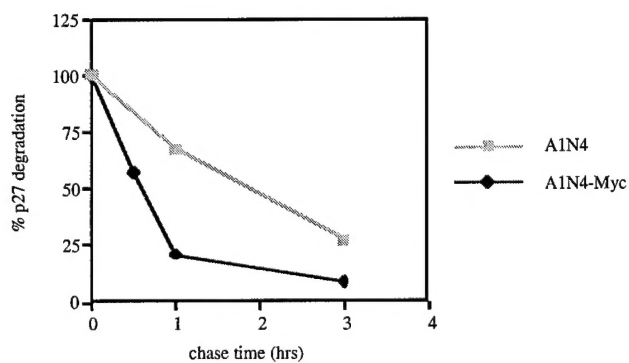
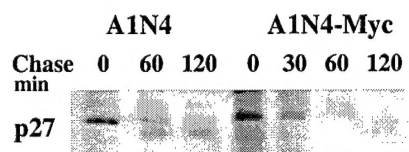


Figure 2: Analysis of the cyclin E-CDK2 complex in non-adherent A1N4 and A1N4-Myc cells. Total cell lysates were prepared from quiescent cells, or cells stimulated with EGF as described in figure 1. A- Total protein were analyzed by immunoblotting with antibodies to cyclin E, CDK2, p27 and p21. B- CDK2 complex was immunoprecipitated with antibody to CDK2 and then separated on SDS-PAGE and immunoblotted with antibodies to p27 and CDK2. C- CDK2 *in vitro* kinase assay. CDK2 complexes were immunoprecipitated with a CDK2 antibody and incubated with [γ^{32} ATP] and histone H1 as a substrate.

A -



B -



C -

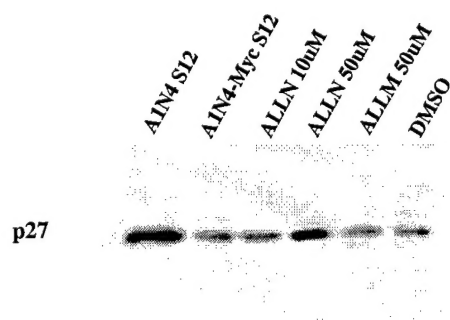


Figure 3: Post translational regulation of p27 in non-adherent mammary epithelial cells. A- Total RNA isolated from adherent and suspended A1N4 and A1N4-Myc cells were analyzed by Northern blot. B- p27 stability was analyzed by pulse chase experiment. A1N4 -Myc cells in suspension were labeled for one hour with [35 S]methionine and then chased in non-radioactive medium for up to 120 min. p27 was then immunoprecipitated from total cell lysates. C- Effect of proteasome inhibitors on p27 degradation. A1N4-Myc cells were grown in suspension for 12 hours alone, or in the presence of the proteasome inhibitor ALLN the calpain inhibitor ALLM, or the DMSO vehicle.